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Composites of Crosslinked Aggregates of Eversa® Transform and Magnetic Nanoparticles. Performance in the Ethanolysis of Soybean Oil

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Abstract: Eversa® Transform 2.0 has been launched to be used in free form, but its immobilization may improve its performance. This work aimed to optimize the immobilization of Eversa® Transform 2.0 by the crosslinked enzyme aggregates (CLEAs) technique, using almost all the available tools to improve its performance. Several variables in the CLEA preparation were optimized to improve the recovered activity, such as precipitant nature and crosslinker concentration. Moreover, some feeders were co-precipitated to improve the crosslinking step, such as bovine serum albumin, soy protein, or polyethyleneimine. Starch (later enzymatically degraded) was utilized as a porogenic agent to decrease the substrate diffusion limitations. Silica magnetic nanoparticles were also utilized to simplify the CLEA handling, but it was found that a large percentage of the Eversa activity could be immobilized on these nanoparticles before aggregation. The best CLEA protocol gave a 98.9% immobilization yield and 30.1% recovered activity, exhibited a porous structure, and an excellent performance in the transesterification of soybean oil with ethanol: 89.8 wt% of fatty acid ethyl esters (FAEEs) yield after 12 h of reaction, while the free enzyme required a 48 h reaction to give the same yield. A caustic polishing step of the product yielded a biodiesel containing 98.9 wt% of FAEEs and a free fatty acids content lower than 0.25%, thus the final product met the international standards for biodiesel. The immobilized biocatalyst could be reused for at least five 12 h-batches maintaining 89.6% of the first-batch yield, showing the efficient catalyst recovery by applying an external magnetic field.

Keywords: lipases; Eversa; magnetic CLEAs; immobilization; ethanolysis; biodiesel

1. Introduction

The current concern about global warming and the reduction of fossil fuel sources have driven the search for less polluting and renewable fuels [1–6]. Biodiesel, obtained by esterification of fatty acids or transesterification of vegetable oils and animal fats with short-chain alcohols, has become an attractive alternative combustible [2–7].

Currently, the production of biodiesel at a large scale uses acids or alkalis as catalysts [4,8], but the replacement of chemical catalysts by enzymatic ones has been widely studied [6,7,9–11]. The enzymatic production of biodiesel can overcome some drawbacks of the chemical route, such as high energy demand, the requirements of raw material with high purity (e.g., anhydrous alcohols and low acid oils), complexity of the recovery and purification of the product, and the necessity of effluent treatment [7–10,12]. However, the main disadvantages of the use of lipases are the higher biocatalyst

cost and the longer reaction time compared to the chemical route [13–16]. Another problem of lipases is that they have substrate specificity, thus one specific lipase may not recognize the high number of likely substrates contained in an oil, which is an heterogeneous substrate [17,18].

The enzymes used in biodiesel production are lipases (triacylglycerol acyl hydrolase, EC 3.1.1.3) [8,13,19]. The natural function of these enzymes is the hydrolysis of triglycerides at the water-oil interface, releasing free fatty acids, diglycerides, monoglycerides, and glycerol. However, they are also able to catalyze esterification and transesterification reactions in organic media (with restricted water content) [11,16,20]. Lipases have two different conformations due to the presence of a lid-acting polypeptide chain. Their interaction with a hydrophobic surface causes the enzyme to move from its inactive (closed lid) to active (open lid) conformation, thus exposing its active site, conferring catalytic activity and free access to the substrate. This lipase catalytic mechanism is known as an interfacial activation [21–23]. This mechanism has been used to develop protocols that enable the one step immobilization, purification, stabilization, and hyperactivation of lipases by immobilizing their open form on hydrophobic supports [24,25].

Eversa® transform was announced in 2014 as the first commercially available liquid enzymatic formulation for biodiesel production, and in 2016 a new version was launched to the market, Eversa® Transform 2.0 [26]. Both are genetically modified variants of the lipase from *Thermomyces lanuginosus* with an improved thermal stability [27,28]. These formulations have been evaluated mainly in their liquid form in the production of biodiesel with good yields (83%–97%) [29–36]. However, their use in an immobilized form has been more scarcely reported [37–40]. Recently, the comparison between immobilized Eversa® Transform 2.0 and lipase from *Thermomyces lanuginosus* showed that, although both enzymes should be similar in their sequence, their functional properties could be fairly different, and this may promote the fact that the same treatment for both enzymes may offer quite different results [41].

Enzyme immobilization, if properly designed, may increase the biocatalyst operational stability, facilitating its recovery, and allowing its reusability [24,25,42–47]. However, the cost of these supports can increase the price of the final biocatalyst. As an alternative to the use of solid supports, Professor Sheldon developed a very simple carrier-free immobilization technique, the crosslinked enzyme aggregates (CLEAs) [48–54].

The crosslinked enzyme aggregates (CLEAs) enzyme immobilization technique permits producing a biocatalyst with high volumetric activity, does not require highly pure enzymes solutions, and allows the co-immobilization of different enzymes [49]. However, it has some problems, such as low mechanical resistance and high diffusion limitations [44]. To prepare a CLEA, the protein is precipitated by the action of a precipitating agent (e.g., salts, organic solvents, polymers, etc.), followed by a chemical crosslinking with bifunctional (usually glutaraldehyde) or polyfunctional (e.g., polyaldehyde dextran) agents [55], which react mainly with amino groups of the lateral chain of the lysine residues on the surface of the enzyme [49,56,57]. CLEAs of several lipases have been reported in the literature [53,58,59], including reports by our group [18,60,61]. However, as far as we know, there are no reports in the scientific literature on immobilizing Eversa Transform as CLEAs.

One of the problems in preparing CLEAs is related to the amount of amino groups in the enzyme surface available for an efficient chemical crosslinking. A small amount of these residues makes the crosslinking weak, leading to enzyme leaching [51]. To overcome this problem, the enzyme can be co-aggregated with lysine-rich proteins, such as bovine serum albumin (BSA) [59,62–67], soy protein [60,61,64,68], or even amino-rich polymers, such as polyethyleneimine (PEI) [61,65,66,69–71].

Mass transfer limitations are one of the main drawbacks of CLEAs due to the low porosity of their highly compact supramolecular structures [44,72,73]. Some strategies have been reported to increase the pore size of the CLEAs particles aiming to reduce or prevent diffusion problems. The formation of large pores assisted by a porogenic agent was firstly studied by Wang et al. [74], who co-precipitated the target enzyme with starch, followed after crosslinking by starch degradation by an α-amylase and washed away from the CLEAs. This strategy allowed producing CLEAs with larger pores compared

to traditional CLEAs, thus reducing internal mass transfer limitations and increasing the catalytic efficiency. Recently, Guimarães et al. [61] also evaluated starch as a porogenic agent in the preparation of CLEAs of porcine pancreas lipase. They reported an increase in the expressed activity of the biocatalyst after hydrolyzing the starch with an α-amylase.

Another problem of using CLEAs is their recovery from the reaction medium because of their small size and low mechanical resistance [44]. An easier recovery of CLEAs from the reaction medium can be achieved by co-aggregation of the enzymes with magnetic nanoparticles, resulting in a magnetic CLEA that can be easily separated from the medium by applying an external magnetic field [61,65,75,76]. In addition, the size and functionalization of magnetic particles can determine the final properties of magnetic CLEAs [77]. In this sense, supports bearing acyl and amino groups have been proposed to be very adequate for lipase immobilization [78]. The acyl groups can permit the interfacial adsorption of the enzyme or the promotion of some favorable environment of the lipase [24,25], and the amino groups, together with permitting the ion exchange of the enzymes, may permit including the nanoparticle in the covalent structure of the CLEA.

In this context, this work aims to prepare Eversa CLEAs with magnetic properties to be used in the synthesis of biodiesel by transesterification of soybean oil with ethanol. Among the set of parameters evaluated for CLEA preparation, we evaluated the concentration and the nature of the precipitating agent, the concentration of glutaraldehyde as a crosslinking agent, and the use of protein or polymer co-feeders or magnetic nanoparticles functionalized with amino and octyl groups, and starch to improve the crosslinking step and avoid or reduce mass transfer problems. The functional properties of the best biocatalyst were further characterized.

To meet international standards, biodiesel (B100) must have a min. 96.5 wt% of fatty acids esters, max. 0.8 wt% of monoglycerides (MAGs), 0.2 wt% of diglycerides (DAGs) and triglycerides (TAGs), and max. 0.25 wt% of free fatty acids (FFAs) [2,79,80]. Nielsen et al. [30] reported two strategies to increase the percentage of esters in the biodiesel, aiming at producing an enzymatic biodiesel within those specifications. The first strategy consisted of the esterification of the residual FFAs with the enzyme Lipozyme CALB-L (liquid formulation of the lipase B from *Candida antarctica*), which allowed the reduction of FFAs from 1.2% to <0.25% after 17 h of reaction, and at the same time, MAGs and TAGs contents were also significantly reduced. The second strategy consisted of a direct caustic polishing in the reaction mixture (still with excess alcohol) to neutralize the FFAs, which were after separated as soap by centrifugation. The authors called this the process of one-pot polishing, because it can be carried out in the same transesterification reactor. In this process, FFAs could be reduced to <0.25% after only 30 min of incubation at 60 °C, bound glycerin was reduced to <0.22%, and at the same time, MAGs content was reduced from 0.9% to 0.6%. After polishing, the product phases separated easily due to the absence of an emulsified phase between the biodiesel phase and the heavy phase. Using this process, they reported a percentage of fatty acid esters of 97.5 wt% [30]. Thus, we adopted in this work the caustic polishing of the produced biodiesel to ensure that our product meets the industrial requirements.

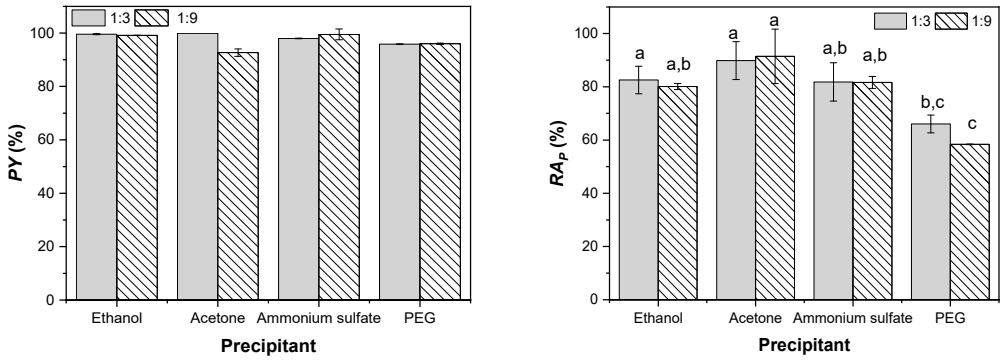
2. Results and Discussion

2.1. Preparation of Eversa-CLEAs

2.1.1. Selection of the Precipitant

All precipitants evaluated were capable of precipitating more than 95% of the enzyme (Figure 1a). However, the precipitate obtained using acetone, ethanol, and ammonium sulfate (saturated solution) were capable of expressing more than 80% of the activity after re-dissolving the precipitates (Figure 1b), while for polyethylene glycol (PEG), the highest recovered activity after re-dissolving the precipitates was 66.0% and 58.4% using an enzyme solution/precipitant ratio of 1:3 and 1:9 (*v*/*v*), respectively. For all precipitants, the increase in their concentration (volume ratio of 1:9) did not show a significant improvement in the recovered activities after re-dissolution. Thus, a volume ratio of 1:3 was selected

for the preparation of Eversa-CLEAs. Ethanol was selected as the precipitant because of its economic and environmental advantages.

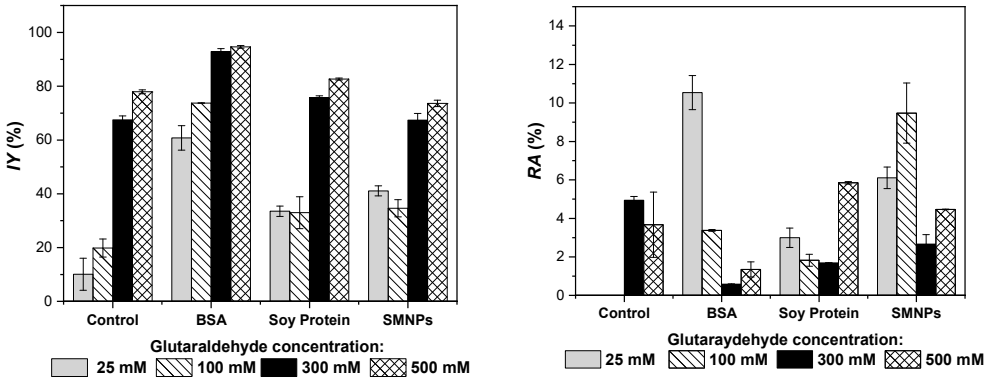


(**a**) (**b**)

Figure 1. Evaluation of the nature and volume ratio enzyme solution:precipitant in the precipitation of Eversa. (**a**) Activity precipitation yield (*PY*, Equation (1)) and (**b**) recovered activity after re-dissolving the precipitates (RA_P, Equation (2)). Precipitation conditions: 4 °C, 150 rpm, 1.5 mg/mL enzyme solution in a 5 mM sodium phosphate buffer, pH 7. Note: Means followed by the same letter are not statistically different by the Tukey test ($p < 0.05$). Hydrolytic activities were measured with tributyrin as the substrate (Section 3.2).

2.1.2. Evaluation of Co-Feeders, Additives, and Glutaraldehyde Concentration in the Preparation of Eversa-CLEAs

Eversa-CLEAs were prepared with and without protein co-feeders (BSA and soy protein) or with silica magnetic nanoparticles (SMNPs) functionalized with amino and octyl groups, using different glutaraldehyde concentrations (25, 100, 300, and 500 mM) in the crosslinking step. Figure 2a,b shows the results of immobilization yields (*IYs*, Equation (3), Section 3.4.5) and recovered activities (*RAs*, Equation (4), Section 3.4.5), respectively.



(**a**) (**b**)

Figure 2. (**a**) Immobilization yields (*IY*, Equation (3)) and (**b**) recovered activity (*RA*, Equation (4)) of Eversa-crosslinked enzyme aggregates (CLEAs) prepared without and with protein co-feeders (bovine serum albumin (BSA), soy protein), or with silica magnetic nanoparticles (SMNPs). Experimental conditions: 5 mg/mL of enzyme solution, protein/co-feeder or additive mass ratio of 1:1, precipitation with ethanol (1:3 enzyme solution volume/ethanol volume), 4 °C, 150 rpm, 30 min of precipitation, followed by a 2.5 h treatment with glutaraldehyde. Hydrolytic activities were measured with tributyrin as the substrate (see Section 3.2).

As shown in Figure 2a, Eversa-CLEAs without any co-feeders or additives (CLEAs control) could be obtained for all glutaraldehyde concentrations, but the highest *IYs* were achieved using crosslinker concentrations higher than 300 mM. Using 500 mM, an *IY* of 77.9% was achieved. For the lowest glutaraldehyde concentrations, aggregated enzyme crosslinking could not be sufficiently intense to prepare physically stable CLEAs, allowing enzyme leaching when they are washed and resuspended [52,70,71].

The use of rich-lysine co-feeders improved the *IY* even at low glutaraldehyde concentrations. The use of BSA as a co-feeder allowed an *IY* up to six times higher than that obtained for the CLEAs control using 25 mM glutaraldehyde. In addition, BSA gave better results than soy protein as a protein co-feeder. The *IY* of 94.6% could be reached using 500 mM glutaraldehyde if BSA was co-precipitated with Eversa. On the other hand, the addition of SMNPs (without any additional protein co-feeder) allowed reaching an *IY* of 73.6% using 500 mM glutaraldehyde.

Although Eversa-CLEAs prepared with BSA or SMNPs using 300 or 500 mM glutaraldehyde yielded high *IYs*, the *RAs* for all CLEAs were less than 10% (Figure 2b). The incubation of free enzyme (5 mg/mL of enzyme solution in a 5 mM sodium phosphate at pH 7.0) in the presence of 300 or 500 mM glutaraldehyde at 4 °C and stirred at 150 rpm preserved 64.1 ± 3.1% and 68.6 ± 2.1% of the Eversa initial activity, respectively, suggesting that the chemical modification of the enzyme was not the main factor responsible for the activity drops in the Eversa-CLEAs, but perhaps some diffusional limitations (i.e., the substrate diffusion rate can be reduced due to the CLEA quite small pores diameter [44]) or small enzyme conformational changes. In that case, depending on the precipitant nature or the precipitation conditions, the enzyme may precipitate in an inactive conformation but its activity is restored when re-dissolved. However, when it is crosslinked it will remain in the inactive conformation and display a lower activity [57]. One additional factor to be considered is the fact that while the free enzyme may be exposed to drops of tributyrin, the enzyme molecules immobilized inside the CLEAs particles will be acting only in the soluble fraction of tributyrin (and the solubility of this compound in an aqueous medium is very low [81]). Due to the better results with BSA and SMNPs, they were chosen for further CLEAs preparation assays. SMNPs were used because they allowed recovering CLEAs by the use of an external magnetic field [61,65,75,82]. In addition, because 500 mM glutaraldehyde allowed preparing CLEAs with the highest *IYs*, it was chosen for further assays.

2.1.3. Use of PEI as a Polymeric Feeder and Starch as a Porogenic Agent

The effect of the incubation of the enzyme with PEI before adding the precipitating agent was evaluated in the preparation of CLEAs with or without BSA or SMNPs, in the presence or absence of starch as a porogenic agent [74]. Table 1 shows that high *IYs* were achieved for all evaluated conditions (more than 97%). Again, Eversa-CLEAs prepared without any additive showed a very low *RA* (less than 1%). However, when the CLEAs were prepared with PEI, the *RA* increased 16.5 times (without any additional additive). Additionally, adding starch and BSA or starch and SMNPs, a small increase was observed on the *RAs*. However, when starch and SMNPs in a nanoparticles:enzyme mass ratio of 3:1 were added in the CLEA preparation, the *RA* increased by a further 2.3 factor.

Regarding the use of starch as a porogenic agent [74], SEM images showed a more porous structure for Eversa-CLEA prepared in the presence of SMNPs (hereinafter named Eversa-mCLEA) (Figure 3a) after starch hydrolysis, while Eversa-CLEA prepared in the presence of BSA (hereinafter named Eversa-BSA-CLEA) (Figure 3b) had a more dense structure. The differences on the CLEAs structures may probably be related to the fact that the enzyme is partially adsorbed on the SMNPs prior to the precipitation with ethanol. In fact, it was checked that under the conditions used in the CLEAs preparation, around 50% of the enzyme activity was adsorbed on SMNPs after 30 min of incubation (Figure S1, Supplementary Data). Thus, Eversa-CLEAs prepared with SMNPs is a mixture of aggregates of non-adsorbed enzyme molecules and SMNPs bearing some Eversa immobilized molecules.

Table 1. Effect of polyethyleneimine (PEI), starch, and the additives bovine serum albumin (BSA) or silica magnetic nanoparticles (SMNPs) on the immobilization yields (*IYs*) and recovered activities (*RAs*) of Eversa-CLEAs, and specific esterification activities of the biocatalysts.

Biocatalyst	*IY* (%)	*RA* (%)	Esterification Activity μmol/min/g of Biocatalyst
1. Liquid Eversa			106.4 ± 10.2
2. Eversa-CLEA in the absence of any additive	99.8 ± 0.1	0.8 ± 0.1	17.9 ± 2.1
3. Eversa-CLEA in the presence of PEI	97.9 ± 0.1	13.2 ± 0.2	95.0 ± 1.5
4. Eversa-CLEA in the presence of PEI and starch	98.2 ± 0.4	15.0 ± 1.6	63.4 ± 6.8
5. Eversa-CLEA in the presence of BSA [a], PEI, and starch	98.3 ± 0.3	17.5 ± 3.2	47.5 ± 8.8
6. Eversa-CLEA in the presence of SMNPs [b], PEI, and starch	98.2 ± 0.4	17.8 ± 0.2	170.9 ± 22.2
7. Eversa-CLEA in the presence of SMNPs [c], PEI, and starch	98.9 ± 0.3	30.1 ± 3.6	106.2 ± 12.4

[a] Enzyme/BSA mass ratio of 1:1; [b] enzyme/SMNPs mass ratio of 1:1, [c] enzyme/SMNPs mass ratio of 1:3. *IYs* were calculated using hydrolytic activities (using tributyrin as the substrate, Equation (3), Section 3.4.5) and *RAs* were calculated using esterification activities (initial rate of butyric acid consumption in the synthesis of butyl butyrate, Equation (4), Section 3.4.5). All CLEAs were prepared using ethanol as the precipitant (enzyme solution:precipitant volume ratio of 1:3) and 500 mM glutaraldehyde in the crosslinking step. The values are expressed as the mean of duplicates ± standard deviation.

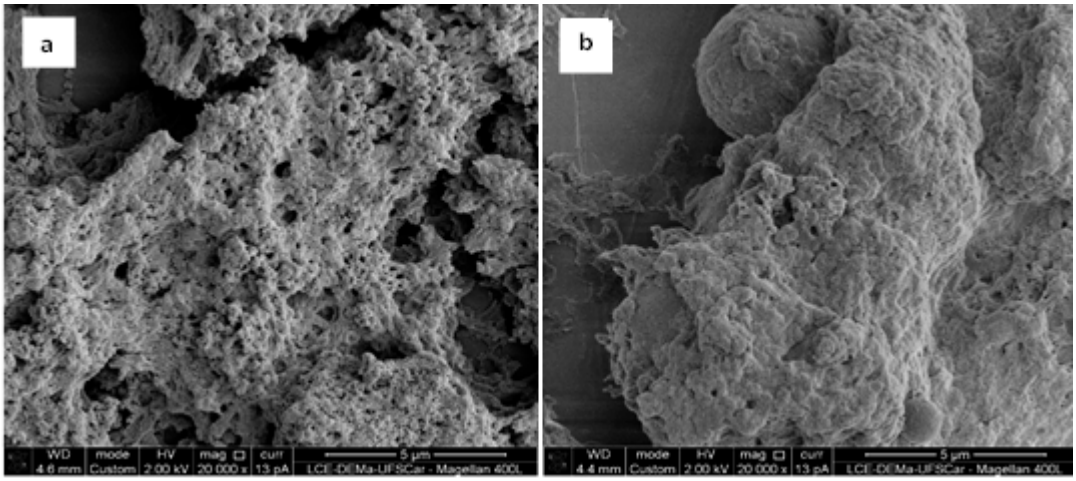


Figure 3. SEM images of Eversa-CLEAs co-aggregated with polyethyleneimine, starch, and (**a**) silica magnetic nanoparticles (nanoparticles/enzyme mass ratio of 1:1) or (**b**) bovine serum albumin. The CLEAs were prepared under the same conditions.

Due to its more developed porosity, Eversa-mCLEA had higher mass activity (2.2 to 3.6 times higher than Eversa-BSA-CLEA). This should be added to its advantage in terms of magnetic properties, e.g., ease of recovery. Thus, Eversa-mCLEA (enzyme:SMNPs mass ratio of 1:3, entry 7, Table 1) was selected for next studies because of its higher recovered activity (30.1%), as well as its faster separation and recovery from the medium because of its magnetic response.

2.2. Characterization of Eversa-CLEAs

The effect of temperature and pH on the activity of the Eversa-mCLEA was compared with those of the free enzyme (Figures 4 and 5).

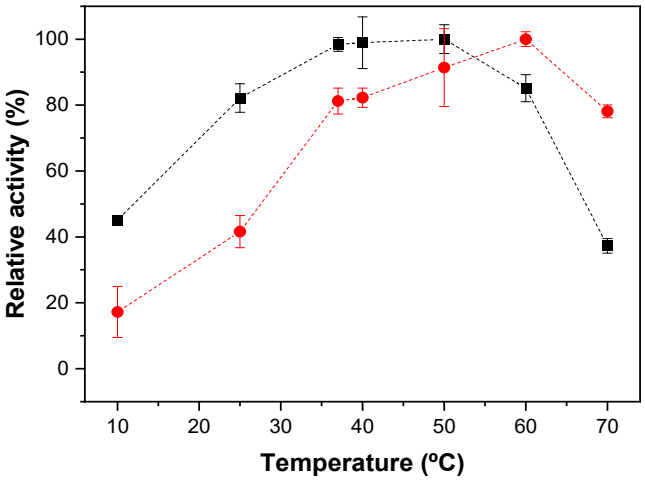


Figure 4. Effect of the temperature on the activities of (■) liquid Eversa and (●) Eversa-mCLEA. The activity was determined by tributyrin hydrolysis (soluble enzyme concentration of 5 mg/mL or 10 mg of Eversa-mCLEA, pH 7.0, 100 mM sodium phosphate buffer).

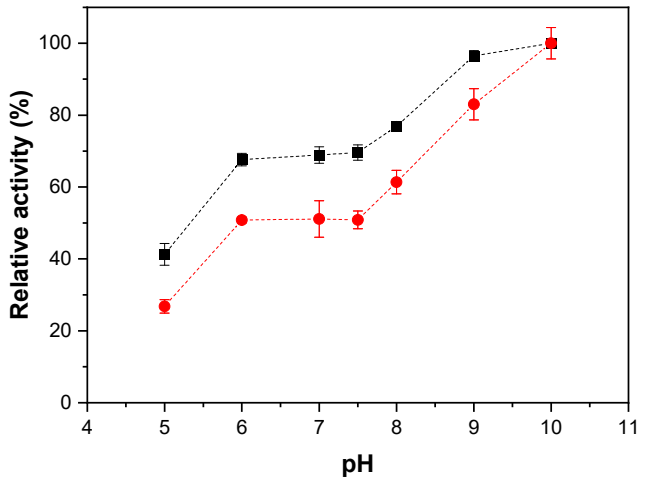


Figure 5. Effect of the pH on the activities of (■) liquid Eversa and (●) Eversa-mCLEA. The activity was determined by tributyrin hydrolysis (soluble enzyme concentration of 5 mg/mL or 10 mg of Eversa-mCLEA, pH 7.0, 100 mM sodium phosphate buffer).

The free enzyme increased the activity up to 40 °C, then it has a wide plateau in the temperature range 35–50 °C, and at 60 °C the activity decreased, and at 70 °C only 40% of the maximum activity was detected. Eversa-mCLEA increased the optimal temperature to 60 °C and at 70 °C, the immobilized enzyme retained around 80% of its maximum activity (Figure 4). This could be associated to an increase in the enzyme rigidity [83–85], as the CLEA is partially formed by Eversa molecules immobilized via interfacial activation (and this already produced some lipase stabilization [25]) and then, the crosslinking with glutaraldehyde may further improve the enzyme stability, with this crosslinking occurring in the enzyme immobilized in the nanoparticles or just in the aggregated enzyme molecules.

The profiles of activity vs. pH for free and immobilized lipases were much closer, with the enzymes expressing maximum activities at pH 10 (the most alkaline pH value studied) (Figure 5). Similar findings were reported by Bresolin et al. [43] for the NS-40116 lipase (a formulation of *Thermomyces lanuginosus* lipase) and by Arana-Peña et al. [37] for Eversa Transform 2.0.

Liquid Eversa was very stable at 60 °C (half-life around 250 h) even in the presence of 100 mM sodium phosphate [86,87] (Figure S2, Supplementary Data). Thus, thermal stabilities of liquid and immobilized Eversa were compared at 70 °C and pH 7. Figure 6 shows that the immobilized lipase was around 40-times more stable than the liquid enzyme (half-lives around 80 and 2 h, respectively).

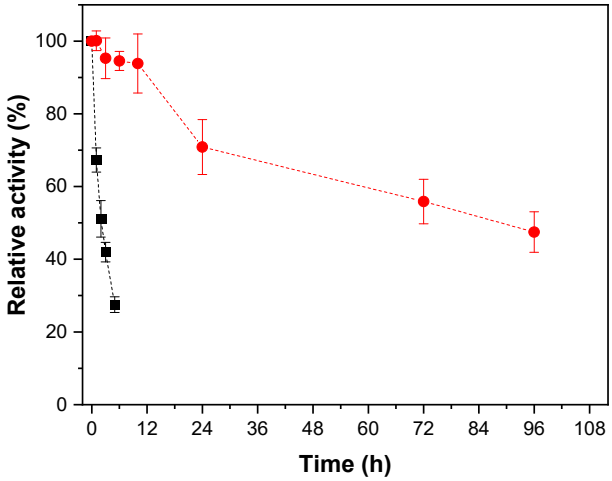


Figure 6. Profile of thermal inactivation at 70 °C of (■) liquid Eversa and (●) Eversa-mCLEA. Assays conditions: Soluble enzyme concentration of 5 mg/mL or 10 mg of Eversa-mCLEA, pH 7.0, 100 mM sodium phosphate buffer. The activity of the enzyme initial solution was taken as 100%. Hydrolytic activities were measured with tributyrin as the substrate.

2.3. Transesterification of Soybean Oil with Ethanol Using Liquid and Immobilized Eversa

Initially, we evaluated the transesterification of soybean oil with ethanol at 35 °C using an oil/ethanol molar ratio of 1:6 and an enzyme load of 3 U_{est}/g oil (equivalent to 3% of liquid Eversa, w/w_{oil}). These conditions were selected based on previous studies with different lipases, including liquid formulations of variants of *Thermomyces lanuginosus* lipases (Callera™ Trans, Eversa® Transform, and NS-40116), in which the temperature ranged from 30 to 45 °C, the oil:alcohol molar ratio ranged from 1:4 to 1:6.3, and the enzyme load ranged from 1.5% to 5% (w/w_{oil}) [88–90].

The concentration profiles of FAEEs, MAGs, DAGs, and TAGs generated in the transesterification reactions, catalyzed by free Eversa (Figure 7a) and the immobilized biocatalysts Eversa-mCLEA (Figure 7b) and Eversa-BSA-CLEA (Figure 7c) were assessed during a full 24 h reaction cycle. For the soluble enzyme and Eversa-mCLEA, the concentration profiles of FAEEs, MAGs, DAGs, and TAGs were very similar. After 24 h of reaction, mostly FAEEs were detected, with a total disappearance of TAGs. However, for Eversa-BSA-CLEA the reaction was slower, with TAGs still corresponding to higher than 70% of its initial concentration, and FAEEs concentration lower than 25% of its maximum value. This reinforced that the best Eversa-CLEA among the ones prepared in this paper was Eversa-mCLEA.

FAEEs yields (mass basis) were also quantified by gas chromatography (Figure 7d) over time. After 24 h of reaction, the free enzyme and Eversa-mCLEA exhibited FAEEs yields of 76.4 and 67.8 wt%, respectively, while Eversa-BSA-CLEA had the worst performance (FAEEs yield of 21.4 wt%). The better performance of the Eversa-mCLEA over the Eversa-BSA-CLEA could be due to the more porous structure of the former (Figure 3) and the presence of hydrophobic groups on the surface of the SMNPs, facilitating the circulation of the hydrophobic triglycerides or due to the presence of 50% of Eversa molecules interfacially activated versus the SMNPs [25] (Figure S1, Supplementary Data).

Based on these results, Eversa-mCLEA was selected for next studies aiming to increase the FAEEs yield, perhaps making the produced biodiesel a fuel (B100) that meets the international standards ASTM D6751 and EN 14214. Thus, next we evaluated the transesterification of soybean oil with ethanol at 40 °C (temperature in which Eversa-mCLEA exhibits around 80% of its maximum activity, Figure 4), and increasing the enzyme load (4, 7, and 12 U_{est}/g oil, equivalent to 3.8, 6.6, and 11.3% of liquid Eversa, w/w_{oil}) to reach the maximum FAEEs yield in a shorter reaction time.

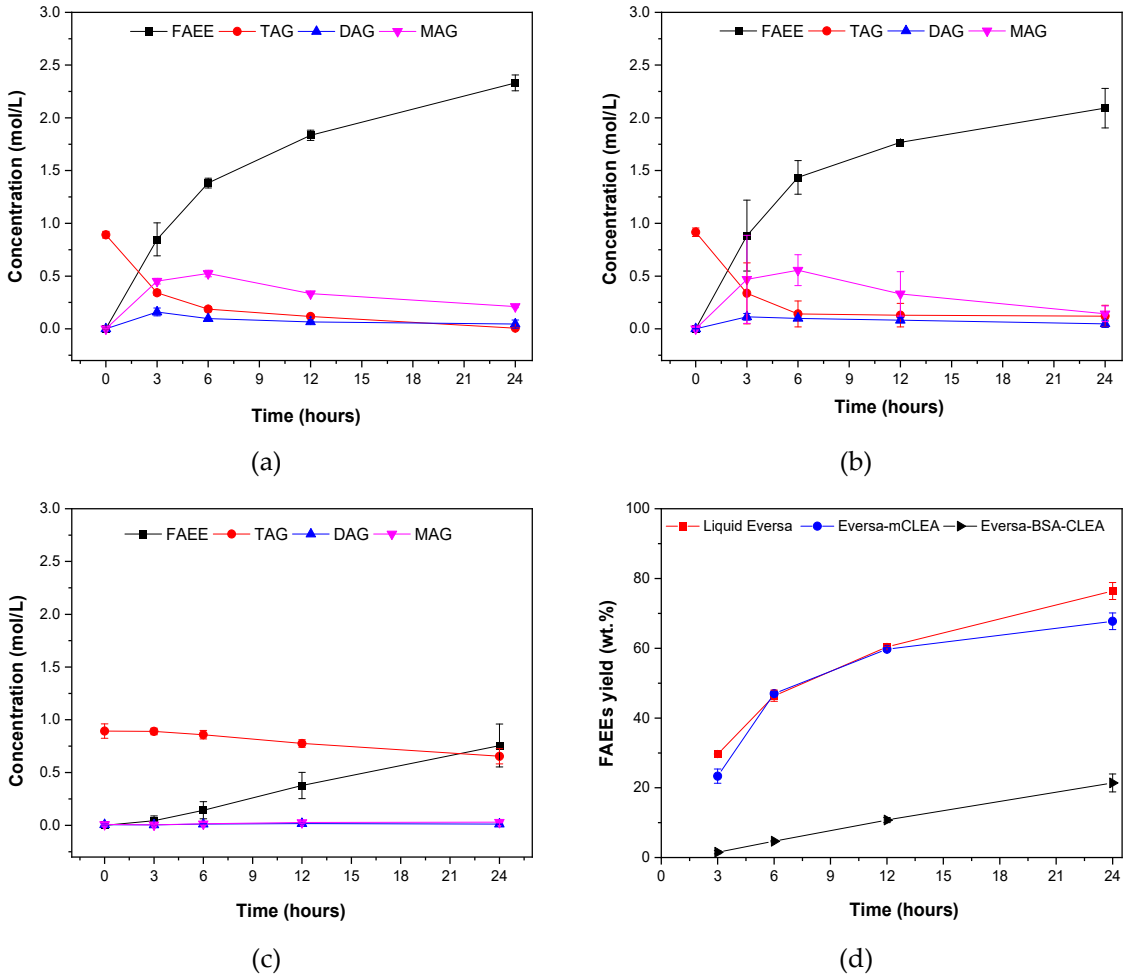


Figure 7. Profiles of molar concentration of fatty acid ethyl esters (FAEEs), triglycerides (TAGs), diglycerides (DAGs), and monoglycerides (MAGs) determined by High Performance Liquid Chromatography with an Ultraviolet Detector (HPLC-UV) (Section 3.9) for the transesterification of soybean oil with ethanol catalyzed by (**a**) liquid Eversa, (**b**) Eversa-mCLEA, and (**c**) Eversa-BSA-CLEA. (**d**) Profiles of FAEEs yield (wt%) determined by Gas Chromatography with a Flame Ionization Detector (GC-FID) (Section 3.10). Reaction conditions: 15 g soybean oil, oil:ethanol molar ratio of 1:6, 3 U_{est}/g oil, 5% (w/w_{oil}) water, 35 °C, and 1500 rpm stirring in a vortex flow reactor. The values are expressed as the mean of triplicates ± SD.

In addition, the liquid Eversa had higher water content (42.6 ± 0.5%) than the Eversa-mCLEA (26.5 ± 0.4%) because of the washing with tert-butanol and dehydration in a refrigerator that formed part of the CLEAs preparation (see Section 3.4.5). Thus, in some cases, the transesterification reactions were performed directly with the biocatalysts, and in others an adjustment of the water content in the reaction catalyzed by the Eversa-CLEAs was performed (Table S1, Supplementary Data).

Figure 8 shows that, using Eversa-mCLEA, the higher the enzyme load, the higher the transesterification initial rate. Using 12 U_{est}/g of oil enzyme load, 87.8 ± 0.7 wt% of the FAEE yield was achieved after 12 h of reaction. After that time, no significant increase was observed in the FAEE yield (89.4 ± 0.1 and 88.5 ± 0.3 wt% after 24 and 72 h of reaction, respectively), but the FFAs content (wt%) increased from 1.19 ± 0.06% (12 h reaction) to 3.8 ± 0.03% (24 h reaction) and 4.06 ± 0.31% (72 h reaction). As this is a kinetically controlled reaction and the FAEE is a substrate of the enzyme [91], some hydrolysis of these esters may be expected if the thermodynamics of the process allows it. Using Eversa-mCLEA, similar initial rates were obtained using 1.6% or 6.9% (w/w_{oil}) water, also reaching similar maximum FAEE yields (around 74–78 wt%) after 72 h of reaction.

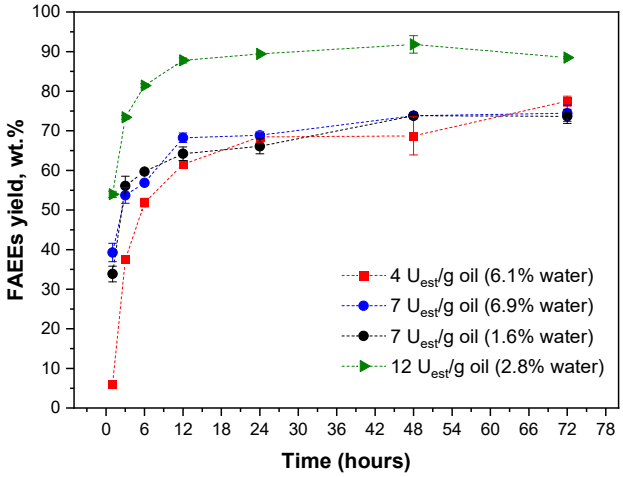


Figure 8. Profiles of FAEE yield vs. time for the transesterification of soybean oil (15 g) with ethanol (oil/ethanol molar ratio of 1:6) catalyzed by Eversa-mCLEA-CLEA at 40 °C and 1700–2000 rpm stirring in a vortex flow reactor.

On the other hand, the reaction catalyzed by liquid Eversa (Figure 9) was very influenced by the water concentration (FAEE yields around 60 and 80 wt% for 2.8% and 6.5% water, respectively), although the initial transesterification rate was not significantly affected. The maximum FAEE yield of around 90 wt% was achieved after 48 h of reaction in the presence of 6.0% water, with a FFAs content of 3.81 ± 0.22 wt%.

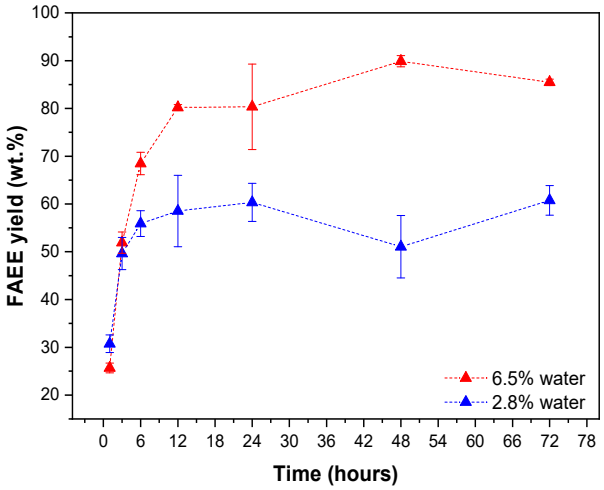


Figure 9. Profile of FAEE yield vs. time for the transesterification of soybean oil (15 g) with ethanol (oil/ethanol molar ratio of 1:6) catalyzed by liquid Eversa at 40 °C and 1700–2000 rpm stirring in a vortex flow reactor using an enzyme load of 7 U_{est}/g oil.

Regarding the use of liquid Eversa, Remonatto et al. [31] reported a yield of 96.7% after 16 h of reaction at 35 °C in the production of methyl esters using soybean oil, 2.5 wt% water, 1.5 methanol equivalent, and 1 wt% liquid Eversa (first formulation version), while Nielsen et al. [30] reported a yield of 85% after 22 h of reaction at 35 °C using refined soybean oil, 0.2% (*w*/*w*) of liquid Eversa, 3% (*w*/*w*) of water, and 1.5 methanol equivalent.

Regarding the use of immobilized Eversa, Bresolin et al. [43] immobilized NS-40116 on polyurethane foam to be used in the transesterification of chicken fat with methanol (fat/methanol mass ratio of 1:9, 2% (*w*/*w*) water, and using an enzyme load of 5% (w/w_{oil})) at 30 °C and for 24 h of reaction, obtaining a FAME yield of 66%. Remonatto et al. [40] immobilized Eversa (second formulation version) on Sepabeads C-18 and used it in the transesterification of sunflower oil (oil/ethanol mass ratio of

1:4, hexane as solvent, and enzyme load of 10%, w/w_{oil}). They reported a FAEEs yield of 99% after 3 h at 40 °C and the reuse of the biocatalyst in four cycles under these conditions showed an activity retention of only 75% of its initial activity.

In this work, the transesterification of soybean oil with ethanol (molar ratio of 1:6) at 40 °C, 2.8% (w/w_{oil}) of water, and 12 U_{est}/g oil (equivalent to 11.3% w/w_{oil} of liquid Eversa) for 12 h gave a FAEEs yield of 87.8 wt% (Figure 8). Despite some different conditions, this value agrees very well with those previously reported. Surprisingly, Remonatto et al. [40] reported the highest FAEEs yield in the transesterification of sunflower oil with ethanol after 3 h of reaction, but in that work, hexane was added to the reaction medium. However, it should be highlighted that biodiesel production is preferentially conducted in a co-solvent-free medium.

The biodiesel obtained in this work, containing 89.8 wt% of FAEEs, 0.27 wt% of MAGs, 0.18 wt% of DAGs, 0.01 wt% of TAGs, and 3.8 wt% of FFAs, was submitted to a caustic polishing to eliminate the excess of FFAs (Section 3.7.2). As a very low amount of NaOH was used, the unwanted effects of adding NaOH to acid oils were significantly reduced. After phase separation and washing with hot water, a biodiesel containing 98.92 wt% of FAEEs, 0.17 wt% of MAGs, 0.19 wt% of DAGs, 0.01 wt% of TAGs, and 0.13 wt% of FFAs could be obtained, which meets the biodiesel international standards (see Introduction Section).

2.4. Reuse Assay of the Magnetic Eversa-mCLEA in Biodiesel Production

Figure 10 shows the reusability of Eversa-mCLEA in the transesterification of soybean oil. The FAEE yields reached in the first and second batches were 86.4 and 84.9 wt%, respectively, decreasing to 76.5 wt% in the third batch, which was maintained until the fifth batch (76.4 and 77.4 wt% for the fourth and fifth batches, respectively). A similar behavior was observed for the FFAs content, which was decreased from 1.2 wt% (first batch) to 0.7 wt% (fifth batch). This probed the easy recovery of the CLEAs, that could be easily recovered using a magnet and they could be well dispersed after washing with tert-butanol. However, part of the activity loss could be derived from the release of some non-magnetic fragments from the magnetic CLEAs after breaking the CLEA particle in the stirring or manipulation [44].

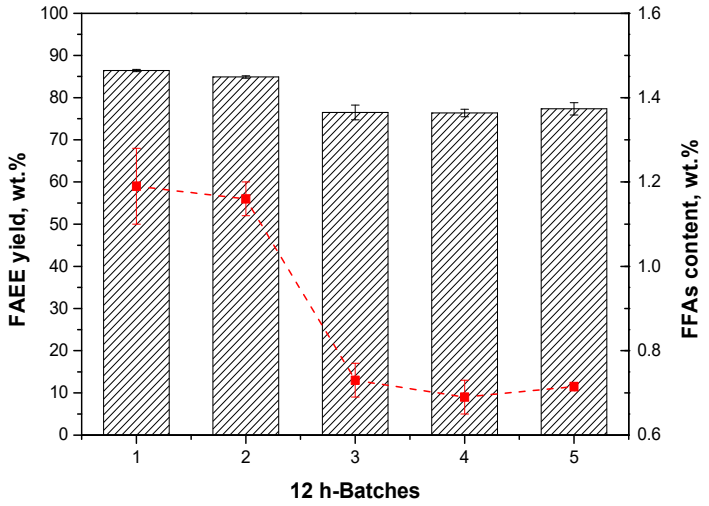


Figure 10. Reuse assay (12 h-cycles) of the Eversa-mCLEA in the transesterification of soybean oil (15 g) with ethanol (oil/ethanol molar ratio of 1:6), enzyme load 12 U_{est}/g_{oil} at 40 °C, and 1700–2000 rpm stirring in a vortex flow reactor. Bars represent FAEE yields and the point-dashed curve represents the FFAs content.

3. Materials and Methods

3.1. Materials

Eversa® Transform 2.0 (a liquid formulation of a variant of the lipase from *Thermomyces lanuginosus* expressed in *Aspergillus oryzae* by Novozymes A/S), BSA, tert-butanol, 97% (*w/w*) tributyrin, PEI, average Mw ~25,000, ≥ 99% (*w/w*) butyric acid, anhydrous 99.8% (*w/w*) 1-butanol, methyl heptadecanoate, polyethylene glycol (PEG), and Bradford reagent were purchased from Sigma-Aldrich (St. Louis, MO, USA). The glutaraldehyde solution (25% *v/v* in H_2O) was purchased from Êxodo Científica (Sumaré, SP, Brazil). Soluble starch and acetone were purchased from Qhemis (Jundiaí, SP, Brazil). Anhydrous ethanol (99.8% P.A.) was purchased from Neon (São Paulo, SP, Brazil). Ammonium sulfate 99% (*w/w*) was purchased from JT Baker (Philadelphia, PA, USA). Soy protein 90% (*w/w*) was acquired from Doremus Ingredientes (Guarulhos, SP, Brazil) and soybean oil (Liza trademark) was from Cargill do Brasil, PR, Brazil. The silica magnetic nanoparticles (SMNPs) functionalized with amine and octyl groups (NanoMag N(75%)-C8(25%)) were purchased from Kopp Technologies (São Carlos, SP, Brazil). According to the supplier, the nanoparticles are non-porous and have 30–120 nm size and approximately 20 m^2 surface area/g of support. All other chemicals and solvents were of analytical grade and they were used as received.

3.2. Enzyme Activity Assays

The hydrolytic activity was measured according to Beisson et al. [92] with minor modifications. A volume of 50 µL of the enzyme solution or CLEA suspension was added to a reaction medium containing 6.0 mL of a 0.1 M sodium phosphate buffer at pH 7.5, 1.5 mL of tributyrin, and 16.5 mL of distilled water. The reaction was carried out at 37 °C, and the reaction mixture was stirred at 500 rpm for 5 min. The hydrolysis of tributyrin was monitored titrimetrically in a Tritrino 907 titrator (Metrohm, Herisau, Switzerland) using a 20 mM KOH solution as the titrating agent. The hydrolytic activity was calculated from the µmols of KOH per minute consumed to neutralize the butyric acid released in the reaction. One tributyrin unit (TBU) was defined as the amount of enzyme that releases 1 µmol of butyric acid per minute under the conditions described. Under these conditions, Eversa® Transform 2.0 (hereinafter named only Eversa) solution had 96,616.60 ± 6556.47 TBU/mL solution (2949.76 TBU/mg protein). The protein concentration of the commercial liquid lipase formulation (32.75 ± 1.75 mg/mL) was determined by the Bradford method [93] using BSA as the standard protein.

The esterification activity was measured in terms of the synthesis of butyl butyrate following the methodology described by Paula et al. [94]. Fifty microliters of soluble enzyme or 50 mg of dried CLEAs were added to the reaction medium containing 7.5 mL of heptane containing 0.1 M butanol and 0.1 M butyric acid and 0.1 g of 3 Å molecular sieves (rod, size 1/16 in, Fluka Analytical, Sigma-Aldrich, St. Louis, MO, USA). The reaction was carried out in 100 mL of closed glass bottles at 37 °C under 250 rpm stirring in an orbital shaker (Model MA832, Marconi, Piracicaba, SP, Brazil). After 60 min of reaction, 5 mL of ethanol were added to quench the reaction, and the acid concentration was measured by titration in a Tritrino 907 titrator (Metrohm, Herisau, Switzerland) using a 20 mM KOH solution. One unit of esterification (U_{est}) was defined as the initial rate of production of butyl butyrate (in µmol/min) under the assay conditions.

3.3. Selection of Precipitants

The precipitation of Eversa was carried out by adding the precipitating agent (acetone, ethanol, PEG or a saturated ammonium sulfate solution) to an enzyme solution (1.5 mg protein/mL in a 5 mM sodium phosphate buffer at pH 7.0) in volume ratios of 1:3 or 1:9 (enzyme solution/precipitant) in an ice bath. The mixture was stirred (150 rpm) for 30 min at 4 °C in an orbital shaker (Model MA830, Marconi, Piracicaba, SP, Brazil), the precipitate was recovered by centrifugation (10,400× *g* at 4 °C for 10 min), the supernatant separated, and the precipitate re-dissolved in 1 mL of a 5 mM sodium phosphate buffer at pH 7.0. The hydrolytic activities (see Section 3.2) in the initial solution (control),

in the supernatant, and in the solution of the re-dissolved aggregates were measured. The precipitation yield (*PY*) and the recovered activity after re-dissolving the precipitate (*RAp*) was calculated using the following equations:

$$PY(\%) = \frac{A_i - A_s}{A_i} \times 100, \quad (1)$$

$$RA_P(\%) = \frac{A_P}{A_i} \times 100, \quad (2)$$

where A_i is the initial total activity offered to the precipitation assay, A_s is the total activity measured in the precipitation supernatant, and A_P is the total activity measured in the re-dissolved aggregates solution.

3.4. Procedure of Eversa-CLEAs Preparation

3.4.1. Standard Assay

An initial solution of Eversa (5 mg protein/mL) was prepared in a 5 mM sodium phosphate buffer at pH 7.0. Ethanol was added to the initial solution to a volume ratio of 1:3 (enzyme solution/ethanol) in an ice bath. The resulting suspension was stirred at 150 rpm at 4 °C in an orbital shaker (Model MA830, Marconi, Piracicaba, SP, Brazil). After 30 min, glutaraldehyde was added to the precipitated enzyme at final concentrations of 25, 100, 300, and 500 mM and for 2.5 h at 4 °C and 150 rpm. Then, the suspension was centrifuged (10,400× *g* for 10 min at 4 °C) and the solid was washed twice with a 100 mM sodium phosphate buffer at pH 7.0 (using the same volume than that of the volume of ethanol added in the precipitation step) at room temperature, and finally re-suspended in a 5 mM sodium phosphate buffer at pH 7.0 using the same volume of the initial solution.

3.4.2. Evaluation of Protein Co-Feeders and Additives

The same procedure described in Section 3.4.1 was used to evaluate the co-aggregation of Eversa with SMNPs as an additive and soy protein or BSA as protein co-feeders. A solution containing SMNPs, BSA, or soy protein to a final concentration of 5 or 15 mg/mL (in a 5 mM sodium phosphate buffer at pH 7.0) was added to the enzyme solution, maintaining mass ratios of 1:1 or 1:3 (enzyme protein/additive), respectively. CLEAs prepared with protein co-feeders were recovered by centrifugation at 10,400× *g* for 10 min at 4 °C. On the other hand, when SMNPs were used, the CLEAs were recovered by applying an external magnetic field (using a neodymium magnet, 50 × 20 × 20 mm).

3.4.3. Evaluation of PEI as a Feeder Polymer in the Eversa-CLEAs Preparation

PEI was evaluated as a feeder polymer aiming to facilitate the crosslinking reaction with glutaraldehyde. The treatment with PEI was performed according to López-Gallego et al. [71], with modifications. An aqueous solution of 75 mg/mL PEI was prepared and the pH was adjusted to 7.0. A volume of this solution was added to the enzyme solution (in a 5 mM sodium phosphate buffer at pH 7.0) for a mass ratio of 1:1 (mg of protein:mg of PEI). This mixture was incubated at 25 °C and stirred in a 3D roller agitator (Kasvi, São José dos Pinhais, PR, Brazil) at 150 rpm. After 60 min, SMNPs or BSA solutions were added to a mass ratio of 1:1 or 1:3 (enzyme /additive) to a final concentration of 5 mg/mL of enzyme, and the other steps followed the CLEA standard preparation described in Section 3.4.1.

3.4.4. Evaluation of Starch as a Porogenic Agent

Starch was added in both the enzyme and the additive (BSA or SMNPs) solutions prepared in a 5.0 mM sodium phosphate buffer at pH 7.0, to a final concentration of 3.2% (*w/v*). Then, ethanol was added to a final volume ratio of 1:3 (enzyme volume/ethanol volume). The other steps followed the standard preparation protocol described in Section 3.4.1. After CLEAs washing (twice using the same volume of ethanol added in the precipitation step) and resuspension (using the same volume of initial solution) in a 5 mM sodium phosphate buffer, 50 µL of α-amylase (BAN 480L), and 50 µL of amyloglucosidase (AMG 300L), both from Novozymes A/S (Bagsvaerd, Denmark), were added,

and the suspension was incubated at 25 °C for 4 h to hydrolyze the starch into dextrins, maltose, and glucose, which can be easily washed away from the CLEAs. Then, CLEAs were recovered by centrifugation or by magnetic separation (if bearing SMNPs), washed twice (using the same volume of initial solution) with a 5 mM sodium phosphate buffer at pH 7, and dried according to the protocol described in Section 3.4.5.

3.4.5. Effect on the Eversa-CLEAs Activity of the Immobilization Parameters

Activity measurements in the initial solution, supernatant after precipitation, washing supernatants, and CLEAs suspension were used to calculate the following immobilization parameters: Immobilization yield (*IY*) and recovered activity (also called expressed activity) (*RA*) [95], using the following equations:

$$IY(\%) = \frac{A_i - (A_s + A_w)}{A_i} \times 100, \quad (3)$$

$$RA(\%) = \frac{A_{CLEA}}{A_i \times IY} \times 100, \quad (4)$$

where the total activities A_i (initial solution), A_{CLEA} (CLEAs suspension), A_s (supernatant), and A_w (washing supernatants) were calculated as the product of the volumetric activity (using tributyrin as the substrate, in TBU/mL, see Section 3.2) and of the solution or suspension volume (in mL). In some cases, *RA* was calculated using the total esterification activities (see Section 3.2) of soluble (offered to the immobilization) and immobilized (measured in the CLEAs) enzymes.

Prior to their use for esterification activity measurement and transesterification reactions, Eversa-CLEAs were washed twice with tert-butanol and kept overnight in a refrigerator in a petri dish for dehydration. After dehydration, the CLEAs were stored in Falcon tubes in a refrigerator for further use. Tert-butanol was chosen because it is non-toxic and it is sufficiently hydrophobic (logP of 0.80) to prevent lipase inactivation [96,97].

3.5. Scanning Electron Microscopy of Eversa-CLEAs

The surface morphology of the Eversa-CLEAs prepared with starch and BSA or SMNPs (named in this work Eversa-BSA-CLEA and Eversa-mCLEA, respectively) were investigated by scanning electron microscopy with field emission gun (SEM-FEG) using a FEI Magellan 400 L (Germany) electron microscope operated at 2 kV.

3.6. Effect of pH and Temperature on Hydrolytic Activity and Thermal Stability of Eversa-CLEAs

The hydrolytic activity of liquid and CLEA formulations of Eversa was determined at different pH values and 37 °C, using 100 mM of different buffers: Sodium acetate at pH 5.0, sodium phosphate at pH values from 6.0 to 8.0, or sodium carbonate at pH 9.0 and 10.0.

To determine the effect of the temperature on the activity of free enzyme and Eversa-CLEA, the hydrolytic activity was measured using a 100 mM sodium phosphate buffer at pH 7.5 in a temperature range from 10 to 70 °C.

For thermal stability assays, free and immobilized enzymes were incubated at 70 °C in a 100 mM phosphate buffer at pH 7.0. At regular time intervals, samples were withdrawn to measure their hydrolytic activity (Section 3.2).

3.7. Transesterification of Soybean Oil with Ethanol Using the Liquid and CLEA of Eversa

The reaction medium was composed of 15 g of soybean oil and 4.75 g of anhydrous ethanol (oil:ethanol molar ratio of 1:6), 1.6–6.6% water (w/w_{oil}), and 3–12 U_{est}/g_{oil} of Eversa in soluble or immobilized (Eversa-BSA-CLEA and Eversa-mCLEA) formulations. A vortex flow reactor [60] was operated at 35–40 °C for 12–72 h and the inner cylinder was rotated at 1500–2000 rpm. Samples were withdrawn to analyze glycerides (monoacylglycerides (MAGs), diacylglycerides (DAGs),

and triacylglycerides (TAGs)), and fatty acid ethyl esters (FAEEs) by liquid and gas chromatography (Sections 3.9 and 3.10, respectively). Water content in the Eversa (liquid and immobilized) formulations was determined by Karl Fisher titrimetric analysis [98] in a Titrino 907 titrator (Metrohm, Herisau, Switzerland). The FFAs contents in the biodiesel products were determined according to the AOCS Official Method Ca 5a-40 (Section 3.8).

3.7.1. Operational Stability of Magnetic Eversa-CLEA in the Transesterification Reaction

Eversa-mCLEA biocatalyst was utilized in successive 12 h-batches of transesterification of soybean oil with ethanol at 40 °C in a vortex flow reactor and stirring at 1700–2000 rpm. In the first batch, the reaction mixture contained refined soybean oil (15 g), anhydrous ethanol to an oil:ethanol molar ratio of 1:6, and 12 U_{est}/g_{oil} of Eversa-mCLEA. After each 12 h-cycle, the biocatalyst was recovered by applying an external magnetic field, followed by washing twice with 10 mL of tert-butanol prior to be used in the next cycle. At the end of each batch, FAEEs, glycerol, TAGs, DAGs, and MAGs were analyzed by gas chromatography (Section 3.10) and FFAs according to the AOCS Official Method Ca 5a-40 (Section 3.8).

3.7.2. Caustic Polishing of Biodiesel

Caustic polishing of biodiesel was carried out according to Nielsen et al. [30], with modifications. After the enzyme catalyzed transesterification reaction and the recovery of the catalyst, the FFAs content of the reaction medium was quantified (Section 3.8), and a volume of 4% (*w/v*) NaOH solution was added to the biodiesel mixture to get the molar proportion of 1.15 molar equivalents per mol of residual FFAs. The mixture was kept at 60 °C in an orbital shaker (Model SL-222, Solab, Piracicaba, SP, Brazil) at 60 rpm for 1 h. Then, the mixture was decanted for 10 min at 60 °C, and then centrifuged at 10,400× *g* for 10 min at 25 °C. The light phase was recovered, washed with hot distilled water (using the same volume of sample), and centrifuged (this protocol was repeated three times). The washed sample was dried overnight in an oven at 60 °C. Then, the dry sample was characterized in terms of FFAs (Section 3.8) and FAEEs, MAGs, DAGs, and TAGs (Section 3.10).

3.8. Free Fatty Acids Determination

FFAs were determined according to the AOCS Official Method Ca 5a-40, but modified by Rukunudin et al. [99]. A sample was mixed with ethanol and titrated in a Tritrino 907 titrator (Metrohm, Herisau, Switzerland) using a KOH solution. The FFAs concentration was calculated as a percentage of oleic acid, according to Equation (5):

$$\%FFAs\ as\ oleic\ acid = \frac{alkali\ volume\ (mL) \times alkali\ normality \times 28.2}{sample\ weight\ (g)} \quad (5)$$

3.9. HPLC Analysis of Biodiesel Production Reactions

The samples from the transesterification reactions were centrifuged at 14,500× *g* for 5 min at 5 °C to separate the two phases (polar and nonpolar phases). Then, 50 µL of the nonpolar phase (oil phase) were weighed and diluted 1650 times in a 2-propanol-hexane solution (5:4, *v/v*). This solution was filtered in a 0.22 µm micropore filter and 20 µL were injected into the device for analysis. The concentrations of TAGs, DAGs, MAGs, and FAEEs were analyzed by liquid chromatography using the methodology proposed by Holcapek et al. [100], with some modifications, in a E-2695 Waters chromatograph (Waters, Millford, CA, USA) equipped with a UV detector (set to 205 nm) using an Ascentis®Express C18 reverse phase column (10 cm × 46 mm × 2.7 µm, Sigma-Aldrich, St. Louis, MO, USA). The mobile phase was composed of water (Phase A), acetonitrile (Phase B), and isopropanol:hexane (5:4, *v/v*) (Phase C) with a flow rate of 1 mL/min. The quantification of the molar concentrations of TAGs, DAGs, MAGs, and FAEEs were calculated by Equation (6) [101]:

$$C_{molar,i} = \frac{\%_{area,i} \times m}{MM_i \times V} \quad (6)$$

where i is a specific component (acylglycerides or FAEEs), $\%_{area\ i}$ is the percentage of chromatographic area of component i, MM_i is the molecular mass of each component i (g/mol), V is the volume of the oil phase sample (L), and m is the sample mass (g).

3.10. Gas Chromatography Analysis of Biodiesel Production Reactions

FAEEs, glycerol, TAGs, DAGs, and MAGs were quantified by gas chromatography in a 7890A Agilent chromatograph (Santa Clara, CA, USA) equipped with a flame ionization detector.

Analyses of FAEEs were performed according to the ASTM D6751 and EN14103 methods [102], with modifications. The injector and detector were set at 250 °C. The separation of FAEEs was carried out in a Rtx-Wax capillary column (30 m × 0.25 mm × 0.25 μm, Restek Corporation, Bellefonte, PA, USA) using a temperature of 210 °C and helium as carrier gas. Methyl heptadecanoate was used as an internal standard. For analysis, 1 mL of the reaction mixture sample was centrifuged at 10,000× g for 10 min at 4 °C. The light phase was recovered, washed with hot distilled water (using the same volume of sample) and centrifuged (three washing steps), and dried overnight in an oven at 60 °C. The dried FAEEs samples (50 mg) were diluted in 1 mL of a methyl heptadecanoate solution (10 mg/mL, in heptane) and 1 μL was injected into the equipment. The FAEEs yield (wt%) was calculated according to Equation (7):

$$\text{FAEEs } yield = \frac{(\sum A) - A_{SI}}{A_{SI}} \times \frac{C_{SI} \times V_{SI}}{m} \times 100, \quad (7)$$

where $\sum A$ is the total peak area of fatty acid ethyl esters C14:0 to C24:0, A_{SI} is the peak area of the internal standard (C17), C_{SI} is the concentration of the internal standard (10 mg/mL), V_{SI} is the volume of the internal standard solution (1 mL), and m is the mass of sample (50 mg).

The contents of free glycerol, TAGs, DAGs, and MAGs (in wt%) were determined by gas chromatography in a 7890A Agilent chromatograph (Santa Clara, CA, USA) equipped with an automatic on-column and a flame ionization detector. A sample volume of 1 μL was injected in a Select Biodiesel column (glycerides, UM + 2 mRG, 15 m × 0.32 mm × 0.1μm, Agilent Technologies, Santa Clara, CA, USA), and the following temperature ramp was used: 50 °C for 1 min, heating to 180 °C at 15 °C/min, 230 °C at 7 °C/min, and 380 °C at 10 °C/min, kept for 10 min. The detector temperature was 380 °C, and helium gas was used as carrier gas. The construction of the calibration curves using diolein, monoolein, and triolein standards, internal standards (butanetriol and tricaprin), and derivatization using N-methyl-N-(trimethylsilyl)trifluoroacetamida (MSTFA) were all from Sigma-Aldrich (St. Louis, MO, USA). Sample preparation, analysis, and quantification were performed according to the ASTM D6584 and EN14105 methods with modifications [79].

4. Conclusions

The CLEAs of Eversa Transform 2.0 have been reported for the first time in this work. The treatment of the enzyme with PEI was effective to increase the expressed activity of the Eversa-CLEAs. The more active biocatalyst was prepared by co-aggregating the PEI-treated lipase with magnetic nanoparticles functionalized with amino/octyl groups in the presence of starch as a porogenic agent. Under these conditions, a biocatalyst was synthesized with 98.9% of an immobilization yield and 30.1% of recovered activity. The performance of this biocatalyst was very similar to that of the soluble enzyme in the transesterification of soybean oil with ethanol, yielding 87.8 wt% of FAEEs after 12 h of reaction. The immobilized lipase could be reused in five 12 h-batches of soybean oil ethanolysis, maintaining 89.6% of the FAEEs yield of the first batch. A caustic polishing of the produced biodiesel allowed preparing a biodiesel product within the specifications required for a B100 biodiesel as to the required FAEEs, residual glycerides (MAGs, DAGs, and TAGs), and FFAs contents.

Supplementary Materials: The following are available online at http://www.mdpi.com/2073-4344/10/8/817/s1, Figure S1: Adsorption profile of Eversa Transform 2.0 treated with polyethyleneimine on silica magnetic nanoparticles (SMNPs) functionalized with octyl and amine groups; Figure S2: Profile of inactivation of liquid Eversa® Transform 2.0 at 60 °C and pH 7.0 (100 mM sodium phosphate buffer) using an enzyme concentration of 5 mg protein/mL; Table S1: Experimental conditions (enzyme load and water content) for transesterification reactions using liquid and immobilized Eversa.

Author Contributions: Conceptualization, R.C.G., R.F.-L., and P.W.T.; methodology, L.P.M. and J.R.G.; investigation, L.P.M.; data curation, L.P.M., P.W.T., and R.F.-L.; writing—original draft preparation, L.P.M.; writing—review and editing, R.C.G., P.W.T., and R.F.-L.; supervision, P.W.T. and R.-F.L.; funding acquisition, R.C.G., P.W.T., and R.F.-L. All authors have read and agreed to the published version of the manuscript.

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